Comparison of the Neuroapoptotic Properties of Equipotent Anesthetic Concentrations of Desflurane, Isoflurane, or Sevoflurane in Neonatal Mice

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ABSTRACT

Background: Volatile anesthetics facilitate surgical procedures and imaging studies in millions of children every year. Neuronal cell death after prolonged exposure to isoflurane in developing animals has raised serious concerns regarding its safe use in children. Although sevoflurane and desflurane are becoming more popular for pediatric anesthesia, their cytotoxic effects have not been compared with those of isoflurane. Accordingly, using newborn mice, the current study established the respective potencies of desflurane, isoflurane, and sevoflurane and then compared equipotent doses of these anesthetics regarding their effects on cortical neuroapoptosis.

Methods: Minimum alveolar concentrations were determined in littermates (aged 7–8 days, n = 42) using tail-clamp stimulation in a bracketing study design. By using equipotent doses of approximately 0.6 minimum alveolar concentration, another group of littermates was randomly assigned to receive desflurane, isoflurane, or sevoflurane or to fast in room air for 6 h. After exposure, animals (n = 47) were euthanized, neocortical apoptotic neuronal cell death was quantified, and caspase 3 activity was compared between the four groups.

Results: The minimum alveolar concentration was determined to be 12.2% for desflurane, 2.7% for isoflurane, and 5.4% for sevoflurane. After a 6-h exposure to approximately 0.6 minimum alveolar concentration of desflurane, isoflurane, or sevoflurane, neuronal cell death and apoptotic activity were significantly increased, irrespective of the specific anesthetic used.

Conclusions: In neonatal mice, equipotent doses of the three commonly used inhaled anesthetics demonstrated similar neurotoxic profiles, suggesting that developmental neurotoxicity is a common feature of all three drugs and cannot be avoided by switching to newer agents.

What We Already Know about This Topic

- Neuronal cell death after prolonged exposure to isoflurane in developing animals has raised concerns regarding its safe use in children, but its cytotoxic potency has not been directly compared with the more contemporary agents desflurane and sevoflurane

What This Article Tells Us That Is New

- In neonatal mice, similar neurotoxic profiles were observed using equipotent doses of the three inhaled anesthetics, suggesting that developmental neurotoxicity is a common feature of all three drugs

RECENT data obtained in newborn animals have raised serious safety concerns regarding current anesthesia practice in young children. Numerous studies in small rodents, guinea pigs, and rhesus monkeys have demonstrated a widespread increase in neuroapoptosis shortly after exposure to a variety of anesthetics; some investigations have observed long-term impairment of neurologic function (reviewed by Loepke and Soriano[1]). Neuronal apoptosis, or programmed cell death, plays an integral part in normal brain development, eliminating up to 50–70% of developing neurons.[2,3] This process establishes proper central nervous system structure and function,[4] removes brain cells after pathologic insults (*e.g.*, hypoxia and ischemia),[5] and represents the primary cell death mechanism eliminating neurons after anesthetic exposure in immature animals.[6] The executioner cysteine protease, caspase 3 (the principal marker for apoptotic cell death), is positioned at the center of the apoptotic cascade of proteolytic enzymes. Substantially increased caspase 3 expression was recently demonstrated after 6-h anesthetic exposure to isoflurane in several brain regions, with

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the superficial neocortex being one of the regions consistently exhibiting substantial apoptotic cell death.[7] Although isoflurane's neuroapoptotic properties have been described repeatedly in immature animal models, few studies have examined the other inhalational agents commonly used during pediatric anesthesia, sevoflurane, and desflurane. More important, there is no direct comparison of the three inhaled anesthetics in neonatal animals. Moreover, the relative potencies of these three volatile anesthetics in neonatal mice remain unknown. Accordingly, to help determine whether there is any advantage in using one particular anesthetic, the current study in neonatal mice determined the minimum alveolar concentrations (MACs) of desflurane, isoflurane, and sevoflurane; subsequently, equipotent doses of these anesthetics regarding their effects on cortical neuroapoptosis were compared in neonatal mouse littermates.

Materials and Methods

Subjects

All procedures were approved by the Institutional Animal Care and Use Committee of the Cincinnati Children's Research Foundation, Cincinnati, Ohio; and conformed to the guidelines for ethical treatment of animals. Efforts were made to minimize the number of animals used. Breeding pairs consisting of male CD1 and female C57BL6/J mice were housed on a 12/12-h light/dark cycle at 22°C with free access to food and water. All experiments included both male and female pups (n = 89) on postnatal days 7–8, with the day of birth considered as postnatal day 0. This particular hybrid strain was selected because previous studies[7] demonstrated that these animals exhibit robust anesthesia-induced cell death, with mortality lower than 20% during a 6-h anesthetic exposure.

Experiment 1: Determination of MAC

To determine the relative potency of desflurane, isoflurane, and sevoflurane in neonatal mice, the MAC was quantified using a bracketing study design with tail-clamp stimulation, as previously described.[8] Spontaneously breathing neonatal littermate mice (n = 42) were randomly assigned to receive desflurane, isoflurane, or sevoflurane in oxygen. Animals were separately placed in a 10 × 10 × 25-cm acrylic container, and anesthesia was induced with the respective anesthetic. On loss of the righting reflex, the animals were transferred to a stage warmer (MidAtlantic Diagnostics Inc., Marlton, NJ) set to 36.5°C while inhaling the respective anesthetic *via* a nose cone. Because the animals' small respiratory tidal volume precluded measurements of end-tidal anesthetic concentrations, the inhaled anesthetic concentration was measured (RGM 5250; Datex-Ohmeda Inc., Louisville, CO) and kept constant for 15 min in between stimulations, to allow for equilibration of brain tissue and inspired gas concentrations. After the equilibration period, a supramaximal pain stimulus was generated by clamping the middle third of the animals' tail with a hemostat clamp. Purposeful movement or phonation prompted a 10–15% increase in the anesthetic concentration, whereas lack of response led to a 10% decrease. Any movement other than breathing was considered a response. The new concentration was held constant for 15 min, followed by repeat stimulation proximal to the previous test site to avoid any influence of desensitization of the previously stimulated area on MAC measurements. The MAC was calculated in each animal as the mean of the anesthetic concentrations bracketing the response and lack of response and was averaged for all animals exposed to the same inhaled anesthetic. To exclude interference of other physiologic factors, such as the previously described hypoglycemia, hypercapnia, and metabolic acidosis during prolonged anesthetic exposure in neonatal mice, the number of stimulations was limited to four.[7,8] In a subset of animals, pH, blood gases, electrolytes, and glucose were measured at the end of the MAC determination (n = 18) in blood aspirated from the right carotid artery, as previously described.[8] Blood was analyzed with an analyzer (i-STAT Portable Clinical Analyzer; i-STAT Corp., East Windsor, NJ) using CG8+ cartridges (Abbott Point of Care Inc., Abbott Park, IL). After the experiments, animals were euthanized with an intraperitoneal overdose of a combination of ketamine (20 mg/g), acepromazine (0.5 mg/g), and xylazine (1 mg/g).

Experiment 2: Comparison of Neuroapoptosis after Anesthetic Exposure

Another group of mouse pups was randomly assigned to one of four groups: 6-h exposures to (1) room air (control), (2) 7.4% desflurane, (3) 1.5% isoflurane, or (4) 2.9% sevoflurane in 30% oxygen in a 10 × 10 × 25-cm acrylic container housed in an incubator set at 35.5°C. Anesthetic and carbon dioxide concentrations were continuously monitored (RGM 5250). Control animals were kept in a similar enclosure in a separate incubator. The anesthetic doses represented 0.55–0.6 MAC of the respective anesthetic, as determined in experiment 1. Immediately after the exposure, animals (n = 47) were euthanized and brains were removed and split along the midline. The left hemispheres were used for colorimetric analysis of caspase 3 activity, as described later. The right hemispheres were immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (pH 7.4), postfixed overnight at 4°C, and cryopreserved in 25% sucrose. They were then snap frozen, 40-μm coronal sections were cut on a cryostat (Thermo Electronics, Kalamazoo, MI), and sections were mounted to charged slides and stored at −80°C until use. In a separate group of survivors (n = 28), arterial blood was aspirated and analyzed immediately after the 6-h anesthetic exposure, as described for experiment 1. Control animals were anesthetized with isoflurane for less than 5 min, after the 6-h fasting period, to obtain the blood sample. After blood draw, animals were euthanized, as described for experiment 1.

Immunohistochemistry

Slide-mounted brain sections corresponding to bregma −2.46 to −2.7 mm (figs. 51–53 in Paxinos and Franklin's

mouse brain atlas[9]) were blocked for 1 h in normal goat serum, followed by incubation in rabbit antiactivated caspase 3 polyclonal antibodies (1:100; Cell Signaling, Danvers, MA; 9661 l) for 18 h at 4°C, combined with mouse anti–NeuN monoclonal antibodies (1:100; Chemicon, Billerica, MA; Catalog No. MAB377). Sections were rinsed in blocker and incubated in goat antirabbit (Alexa Fluor 488, 1:250, A11034; Invitrogen Corp., Carlsbad, CA) and goat antimouse (Alexa Fluor 594, 1:250, A11032; Invitrogen Corp.) secondary antibodies for 4 h at 20°C. All antibody solutions were prepared in blocker. After immunostaining, sections were dehydrated in an ascending ethanol series, cleared in xylenes, and mounted with Krystalon (EMD, Gibbstown, NJ). Sections from control and anesthesia-exposed animals were processed simultaneously.

To quantify neuronal apoptosis, two-channel confocal image stacks of NeuN and caspase 3 immunostaining were collected through the *z* depth of the tissue from neocortical brain sections using confocal microscopy (Leica SP5, 63 × 1.4 numeric aperture objective, ×2 optical zoom, 1-μm step; Leica Microsystems, Wetzlar, Germany). Caspase 3 immunostaining was excited using the 488-nm laser line, and emission wavelengths between 517 and 527 nm were collected. Immunostaining for NeuN was excited using the 543-nm laser line, and emission wavelengths between 610 and 660 nm were collected.

Three 120 × 120-μm sampling areas from layers II/III of the right neocortex were scanned at 120-μm intervals, beginning 750 μm lateral from the midline and moving in a lateral direction. Image stacks were then transferred to software (Neurolucida, v7.50.4; MBF Bioscience, Williston, VT) for analysis. An observer unaware of group assignment quantified the number of caspase 3/NeuN double-positive neurons in each field using the optical dissector method while averaging measurements for all three sampling areas to produce a single value for each animal.[7,10–12]

Colorimetric Assay

Activated caspase 3 colorimetric assay kits (Clonetech Laboratories Inc., Mountain View, CA) allow measurement of activated caspase 3 activity by spectrophotometric detection of the chromophore p-nitroaniline (pNA) after cleavage by the enzyme. For analysis, neocortical tissue samples were dissected from the left hemispheric cortex around bregma −2.5 mm and homogenized for 10 s at 4°C in 0.5-ml cell lysis buffer, containing protease inhibitors. The homogenate was then centrifuged for 10 min at 13,000 rpm, and the supernatant was removed and incubated at 37°C for 1 h after the addition of 1 ml Asp-Glu-Val-Asp-pNA solution. The total protein concentration was measured using the Bradford method.[13] Briefly, absorbencies were compared with a standard curve to calculate units of caspase 3. A pNA calibration curve was generated by diluting 100 nM pNA solution in dimethyl sulfoxide to make stock solution of 0, 2.5, 5, 10, and 20 nM. Measurement of the five dilutions with a spectrophotometer (Genova; Jenway, Staffordshire, United Kingdom) at 410 nm created a standard curve of optical density units per pNA concentration. Samples were prepared by measuring volumes equal to 1 μg total protein and combining them with ×2 reaction buffer containing 10 mM dithiothreitol plus Asp-Glu-Val-Asp-pNA, bringing the final concentration of Asp-Glu-Val-Asp-pNA to 50 mM. Samples were read at 410 nm and compared against the slope of the standard curve. Units of caspase activity were then extrapolated from the obtained slope. Caspase units are expressed as nM/μg protein.

Statistical Analysis

Data are presented as mean ± SD for parametric data and as median (95% confidence interval) for nonparametric data, as tested using the Shapiro–Wilk test of normality. If any cell failed to meet the normality criteria, group comparisons were performed using the Kruskal–Wallis test, with a Dunn test for *post hoc* analysis. Mortality was compared among groups using χ^2 contingency table analysis. Calculations were analyzed using computer software (Stata/IC 10.1 for Macintosh; Stata Corp, College Station, TX). Statistical significance was accepted at $P < 0.05$.

Results

The MACs were determined as approximately 12.2% for desflurane, 2.7% for isoflurane, and 5.4% for sevoflurane (fig. 1). Arterial pH, blood gases and chemistry, and hematocrit, as measured after determination of MAC, demonstrated respiratory acidosis, with maintained oxygenation and serum electrolyte concentrations (table 1). All blood parameters were similar among groups, except for a higher glucose concentration in sevoflurane-treated animals compared with animals after desflurane exposure ($P < 0.05$).

By using the determined MAC values, 7–8-day-old mice were exposed to equipotent doses of 0.55–0.6 MAC desflurane, isoflurane, or sevoflurane for 6 h. Control mice fasted in room air for 6 h. Mortality during this prolonged exposure was 16%, 20%, 23%, and 0% for desflurane, isoflurane, sevoflurane, and room air, respectively. Anesthesia-treated groups were statistically equivalent. Arterial pH, base excess, blood glucose, and calcium concentrations were decreased during anesthetic exposure, whereas potassium concentration was increased, compared with animals exposed to room air (table 2), without differences among the anesthesia groups. Neuroapoptosis after anesthetic exposure was quantified by two independent methods: immunohistochemically and a colorimetric caspase 3 assay. The results of the immunohistochemical analysis, using the optical dissector method for quantification of caspase 3–positive neurons (caspase 3/NeuN–positive cells), are displayed in figure 2. All three anesthetic regimens significantly increased caspase 3 expression in neocortical neurons located in layers II/III, compared with unanesthetized littermates that fasted ($P < 0.005$). There were no statistical differences in the quantification of apoptotic neurons among the three anesthesia groups ($P > 0.8$ for all *post hoc* comparisons). Qualitatively, apoptotic

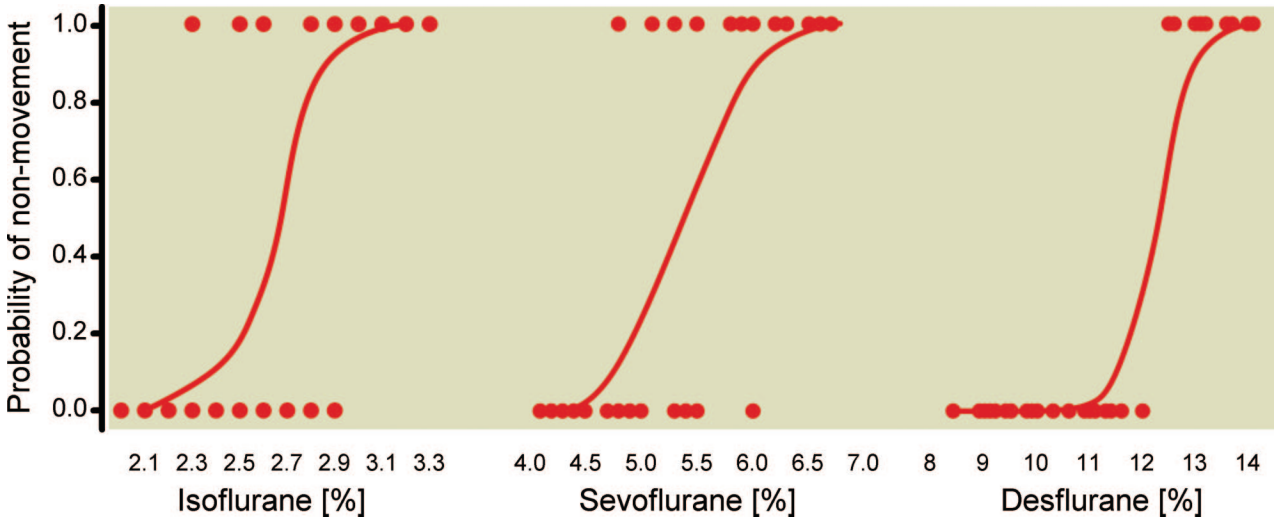

Fig. 1. Probability of nonmovement to tail-clamp stimulation using increasing doses of isoflurane (n = 14), sevoflurane (n = 16), or desflurane (n = 12) during determination of minimum alveolar concentration in 7–8-day-old mice. Movement is plotted as 0; and nonmovement as 1. Each data point indicates one stimulation, with up to three stimulations until nonmovement. The sigmoid curve signifies the probability of nonmovement for the respective anesthetic; inflection points indicate the minimum alveolar concentration for each anesthetic.

neuronal cell death was widespread and appeared similarly distributed among brain regions for all three anesthetic agents (fig. 3). Figure 4 demonstrates a representative section of caspase 3/NeuN colocalization in neocortical neurons affected by anesthesia-induced neuroapoptosis.

In support of these qualitative findings obtained with the immunohistochemical method, a comparative analysis using a colorimetric assay found a significant increase in caspase 3 activity after anesthetic exposure compared with littermates that fasted ($P < 0.0005$), without differences among the three inhaled anesthetics ($P > 0.8$ for all *post hoc* comparisons), as displayed in figure 5.

Discussion

Millions of children are exposed to inhaled anesthetics every year.[14] Serious safety concerns have recently been raised regarding their use during pediatric anesthesia practice based on experiments in newborn animals, in which neuroapoptosis has been observed after prolonged exposure.[15–18] However, most of these animal data relate to one inhaled anesthetic, isoflurane; no direct comparisons have been performed between equipotent doses of isoflurane and the more contemporary inhaled anesthetics desflurane and sevoflurane.

The current study in neonatal mice introduces two key findings: (1) Equipotent doses for the three inhaled anesthet-

Table 1. Minimum Alveolar Concentration for Desflurane, Isoflurane, and Sevoflurane in Neonatal Mice and Blood Gases and Chemistry after 45 Minutes of Exposure to the Inhaled Anesthetics

Variable	Desflurane	Isoflurane	Sevoflurane
Body weight, g*†	4.5 ± 0.8	4.4 ± 0.6	4.4 ± 0.7
MAC, vol%†	12.2 (11.9 to 12.6)	2.7 (2.4 to 2.9)	5.4 (5.0 to 5.9)
RR/min*	23 ± 2	21 ± 2	21 ± 2
pH‡	6.94 (6.75 to 7.04)	7.02 (6.91 to 7.07)	6.92 (6.89 to 6.96)
P_{ACO_2}, mmHg‡	130 (111 to 136)	119 (112 to 128)	130 (130 to 130)
Pa_{O_2}, mmHg‡	451 (298 to 513)	498 (469 to 532)	492 (432 to 543)
BE, mEq/l‡	−2 (−1.2 to −3.2)	−2 (0.8 to −3.2)	−2 (−1 to −2.6)
Na, mEq/l‡	130 (129 to 131)	133 (131 to 136)	131 (127 to 135)
K, mEq/l‡	4.65 (4.48 to 5.02)	5.1 (4.74 to 5.46)	5.1 (4.67 to 5.27)
Ca, mEq/l‡	1.56 (1.37 to 1.7)	1.49 (1.36 to 1.69)	1.56 (1.48 to 1.7)
Hct, %‡	24 (21 to 26)	25 (24 to 26)	26 (23 to 25)
Glucose, mg/dl‡	127 (114 to 148)	143 (109 to 161)	159 (141 to 168)§

Data are given as median (95% confidence interval) unless otherwise indicated.

* Data are given as mean ± SD. † n = 14, 13, and 15 for MAC for desflurane, isoflurane, and sevoflurane, respectively. ‡ n = 6, 5, and 7 for arterial blood gases and chemistry for desflurane, isoflurane, and sevoflurane, respectively. § $P < 0.05$ *vs.* desflurane using the Kruskal–Wallis test with the Dunn test for *post hoc* comparisons (no difference *vs.* isoflurane).

BE = base excess; Ca = serum calcium concentration; Glucose = serum glucose concentration; Hct = hematocrit; K = serum potassium concentration; MAC = minimum alveolar concentration; Na = serum sodium concentration; P_{ACO_2} = partial pressure of alveolar carbon dioxide; Pa_{O_2} = arterial oxygen tension; PRR = respiratory rate.

Table 2. Blood Gases and Serum Chemistry after 6 h of 0.6 MAC of Desflurane, Isoflurane, or Sevoflurane or No Anesthesia in Neonatal Mice

Variable	Desflurane (n = 6)	Isoflurane (n = 7)	Sevoflurane (n = 7)	No Anesthesia (n = 8)*
pH	7.14 (7.06 to 7.2)†	7.1 (7.02 to 7.15)†	7.1 (7.07 to 7.22)†	7.33 (7.24 to 7.41)
P_{ACO_2}, mmHg	74 (61 to 79)†	74 (58 to 86)†	65 (57 to 83)†	50 (42 to 62)
Pa_{O_2}, mmHg	100 (65 to 124)	94 (69 to 135)	136 (88 to 158)	149 (107 to 164)
BE, mEq/l	−5 (−2 to −9.3)†	−9 (−5.7 to −11.4)†	−5 (−2.6 to −7.7)†	0.5 (−1.8 to 2.8)
Na, mEq/l	132 (126 to 134)	134 (132 to 135)	132 (129 to 134)	136 (135 to 137)
K, mEq/l	7.2 (6.8 to 7.5)†	8 (6.7 to 8.5)†	7.9 (7.3 to 8.5)†	4.6 (4.2 to 4.9)
Ca, mEq/l	1.18 (1.11 to 1.26)†	1.25 (1.15 to 1.3)†	1.19 (1.1 to 1.23)†	1.42 (1.37 to 1.46)
Hct, %	34 (27 to 38)	34 (30 to 38)	34 (30 to 36)	32 (28 to 33)
Glucose, mg/dl	37 (28 to 49)†	49 (36 to 60)†	35 (29 to 54)†	74 (56 to 85)

Data are given as median (95% confidence interval).

* Fasting in room air. † $P < 0.05$ *vs.* no anesthesia using the Kruskal–Wallis test with the Dunn test for *post hoc* comparisons (no differences among anesthesia groups).

BE = base excess; Ca = serum calcium concentration; Glucose = serum glucose concentration; Hct = hematocrit; K = serum potassium concentration; MAC = minimum alveolar concentration; Na = serum sodium concentration; P_{ACO_2} = partial pressure of alveolar carbon dioxide; Pa_{O_2} = arterial oxygen tension.

ics were determined by measuring their respective MACs, quantified as 12.2% for desflurane, 2.7% for isoflurane, and 5.4% for sevoflurane. (2) By using clinically significant equipotent anesthetic doses for these three inhaled anesthetics, their neuroapoptotic properties were compared using two independent methods. Both methods demonstrated a robust increase in neuroapoptosis after prolonged exposure to all three agents. Neuroapoptosis was similar in extent and distribution pattern among the three anesthetic agents, demonstrating no particular advantages related to neurotoxicity of using one particular anesthetic over another.

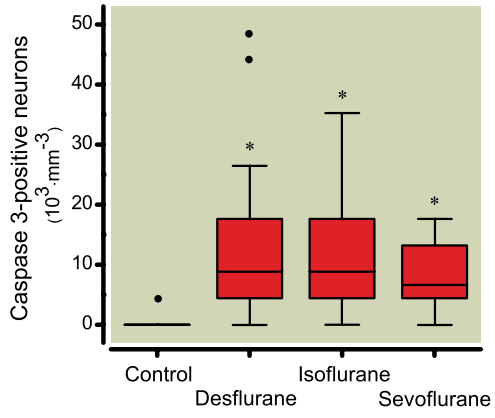

Fig. 2. Exposure to equipotent doses of desflurane, isoflurane, or sevoflurane similarly trigger neocortical neuronal apoptotic cell death in neonatal mice. Box plots represent quantification of neocortical neurons in layers II/III double stained with apoptotic marker activated caspase 3 and neuronal marker neuronal nuclei. Horizontal lines represent respective group medians; boxes, 25th–75th percentile; whiskers, 10^{th}–90^{th} percentile; and closed circles, outliers. The 7–8-day-old mouse pups were treated for 6 h with room air (control, n = 9), 7.4% desflurane (n = 15), 1.5% isoflurane (n = 13), or 2.9% sevoflurane (n = 10). *$P < 0.005$ compared with control. There were no differences among the anesthesia groups ($P > 0.8$), by the Kruskal–Wallis test with the Dunn test for *post hoc* comparisons.

Experiment 1: Determination of MAC

Comparing the deleterious effects of potentially neurotoxic compounds, it is crucial to evaluate comparable doses. Therapeutic comparisons of inhaled anesthetics with differential potencies are commonly made by expressing MAC, the anesthetic concentration needed to prevent movement in 50% of subjects.[19,20] Anesthetic concentration in the spinal cord, the putative target for anesthesia-induced immobility,[20] is most closely approximated by measuring the alveolar anesthetic concentration at the end of expiration. However, because of the small respiratory tidal volume of newborn mice, end-expiratory anesthetic concentrations were not measurable in the current study. Instead, inspired anesthetic concentrations were measured and held constant for 15 min before each stimulus to facilitate equilibration of the anesthetics' inspired and alveolar concentrations, assuming equilibration with the effective site, the spinal cord, similar to previous MAC studies[21] in mice.

It is well-known that MAC can be influenced by several metabolic factors; MAC is decreased by hyponatremia, hypoxia, hypercapnia, hypoglycemia, metabolic acidosis, anemia, and hypoglycemia.[22] Because previous studies[7,8,23] in newborn rodents have demonstrated metabolic and respiratory derangements, such as hypercapnia and hypoglycemia, after prolonged isoflurane exposure, equilibration times were limited to 15 min and the number of stimulations was restricted to four for each animal. Immediately after the last stimulation for MAC determination, no hyponatremia, hypoxia, metabolic acidosis, anemia, or hypoglycemia was observed for any of the three inhaled anesthetics. However, similar degrees of hypercapnia and respiratory acidosis were observed for all anesthetics, similar to previous observations

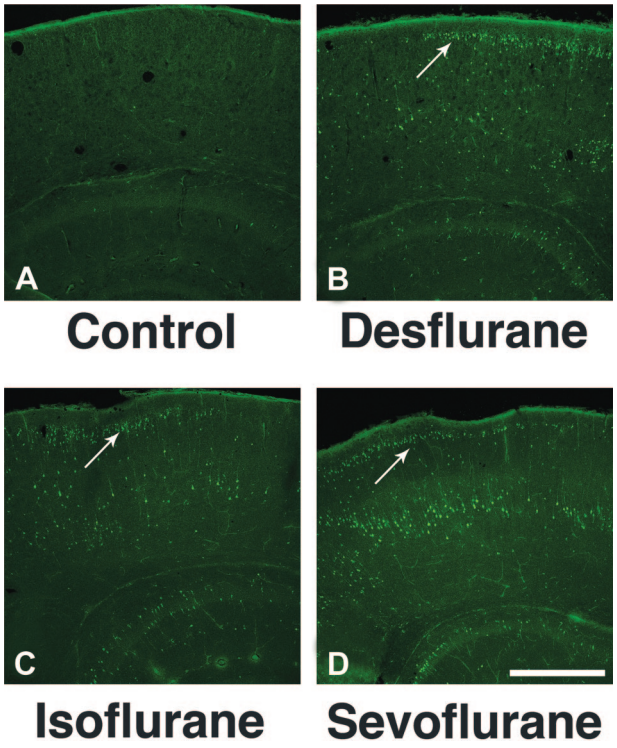

Fig. 3. The 6-h exposure to desflurane, isoflurane, or sevoflurane increases apoptotic cell death in neonatal mice, compared with unanesthetized littermates that fasted. Representative low-magnification photomicrographs of coronal brain sections, obtained with laser confocal microscopy, at bregma −2.46 mm are shown, demonstrating the pattern of neuronal cell death. Brain sections from 7–8-day-old mouse pups were stained for the apoptotic cell death marker activated caspase 3 (bright green) after a 6-h exposure to the following: (*A*) room air (control), (*B*) 7.4% desflurane, (*C*) 1.5% isoflurane, or (*D*) 2.9% sevoflurane. Arrows mark clusters of dying neurons in neocortical layers II/III. The scale bar indicates 500 μm.

for isoflurane in neonatal rats,[23] which could have decreased MAC, albeit similarly, for all anesthetics.

Conversely, after a 6-h exposure to any of the three inhaled anesthetics, significant metabolic and respiratory acidosis and hypoglycemia were observed in all anesthetized animals, compared with controls that fasted. Accordingly, although the inspired anesthetic concentrations remained the same, these metabolic derangements could have decreased MAC and, therefore, resulted in a higher potency of the anesthetic agents. Similarly, acid–base abnormalities and decreases in MAC were previously observed in 7-day-old rats during 4-h exposure to isoflurane,[24] but not in adult rats, which lacked the same respiratory and metabolic derangements.[23] However, contrary to the current results in mice, prolonged isoflurane exposure did not decrease blood glucose concentrations in neonatal rats.[23]

Age has an important effect on MAC, both in animals and humans. Anesthetic requirements are higher in newborn mammals than in adults, increase during infancy, and then decrease into adulthood, reaching a nadir in old age.[25–28] Previous experiments in 10-day-old mice equated the MAC for isoflurane to 2.26%.[8] In adult mice, isoflurane MAC is between 1.2% and 1.4%.[21,29–31] As expected, the current study found a higher MAC of 2.66% for isoflurane in 7-day-old mice, which closely mirrors the MAC of 2.75% recently measured in 7-day-old Sprague-Dawley rats.[24] To our knowledge, MAC concentrations for desflurane and sevoflurane have not yet been reported in neonatal mice. However, similar to isoflurane, MAC concentrations of 5.43%, as measured for sevoflurane, were predictably higher in the 7-day-old mice than the 2.80–3.39% previously reported in adult mice.[30,32,33] Moreover, the MAC of 12.2% for desflurane, as measured in the current study, was also expectedly higher than the 7.01–8.28% previously reported in adult mice.[31,34]

Experiment 2: Comparison of Neuroapoptosis after Anesthetic Exposure

During surgical anesthesia, inhaled anesthetics are often administered in conjunction with muscle relaxants and analgesics, thereby reducing the doses required during the maintenance phase of anesthesia to lower than 1 MAC. By using this paradigm, animals in the current study were exposed for 6 h to 0.55–0.6 MAC of desflurane, isoflurane, or sevoflurane, as determined in experiment 1. Although the exposure time of 6 h was extensive, the doses of 7.4% desflurane, 1.5% isoflurane, and 2.9% sevoflurane were not outside of the range of clinically used doses during pediatric anesthesia.[25–27]

Apoptotic cell death is an integral part of normal brain maturation, eliminating 50–70% of neurons and progenitor cells throughout central nervous system development.[2,3] However, during brain development, neuronal cell death surpassing this natural apoptotic rate can be triggered by pathologic processes, such as hypoxia–ischemia, lack of neurotrophic factors, or prolonged exposure to anesthetics.[1,5] Accordingly, the current study compared the rate of anesthetic-induced neuronal apoptotic cell death using two independent methods, a colorimetric assay measuring caspase 3 expression and an immunohistochemical identification and unbiased cell count of cortical apoptotic neurons, colocalizing NeuN and activated caspase 3.[35] Preliminary unpublished data by our laboratory have suggested that most cortical brain cells affected by apoptotic cell death in 7-day-old mice are postmitotic neurons (not astrocytes or oligodendrocytes).[36] Therefore, the current study is limited to quantification of caspase 3 as the central effector enzyme of the apoptotic cell death cascade in neurons.[37] Researchers have previously demonstrated that caspase 3 expression correlates well with other cell death markers, such as deOlmos silver stain[6,38] and Fluoro Jade B[7] and can, therefore, be used as a marker for anesthesia-induced neuroapoptosis.[39–41] Although anesthesia-induced apoptotic cell death is widespread, affecting most areas of the developing brain, the mechanism underlying the variable selectivity, with dying neurons being surrounded by hundreds of seemingly unaffected cells, has not been resolved. Thus, quantification in

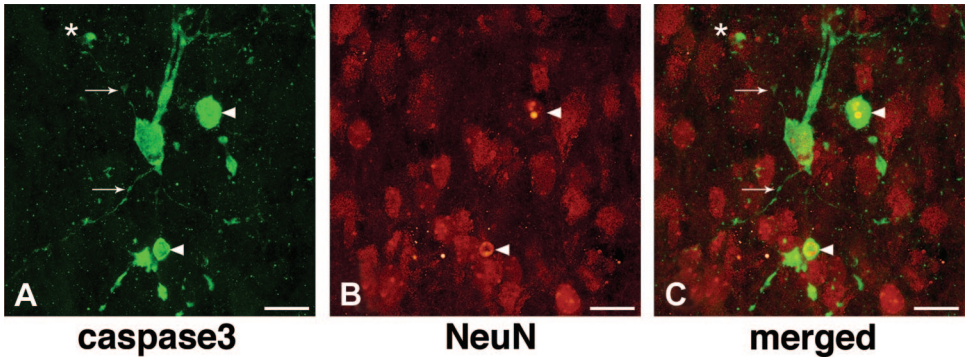

Fig. 4. A representative high-power magnification photomicrograph obtained with laser confocal microscopy, demonstrating colocalization of the apoptotic cell death marker activated caspase 3 (green) and the postmitotic neuronal marker neuronal nuclei (red) in the neocortex. A brain section was obtained from a 7-day-old mouse pup after a 6-h exposure to 1.5% isoflurane, depicting a 9-μm image stack stained for activated caspase 3 (*A*) and NeuN-stained single optical sections through each cell body, for clarity (*B*). Apoptotic neurons, indicated by colocalization of caspase 3 and NeuN (*C*), demonstrate degenerative changes, such as dendritic atrophy (arrows), dendritic beading (*), and pyknotic neurons (arrowheads), and are surrounded by unaffected neurons. The scale bar indicates 10 μm.

the current study focused on a region that has repeatedly exhibited substantial increases in caspase 3–positive cells after anesthetic exposure in small rodents (*i.e.*, the superficial neocortical layers II/III).[7,38] No differences among the three anesthetics were found in this region. The current findings are consistent with previous results demonstrating increased caspase expression after separate exposure to isoflurane[7,42,43] or sevoflurane[44,45]; these findings were expanded to desflurane, which was not studied previously regarding its neuroapoptotic properties. However, our findings differ from recent results demonstrating differential neuronal cell death after sevoflurane exposure compared with isoflurane,[46] which might be explained by the significantly decreased doses for sevoflurane (1.1% *vs.* 2.9%) and isoflurane (0.75% *vs.* 1.5%) used in that study compared with the current study. Moreover, although Liang *et al.*[46] did not specifically measure MAC in their neonatal mice, doses used in their study would equate to significantly decreased doses of 0.2 MAC for sevoflurane and 0.28 MAC for isoflurane, according to our measurements, suggesting that differences in neuroapoptosis may exist among anesthetics at decreased doses.

The mechanism of anesthetic-induced neurotoxicity is not entirely clear. Although it was previously speculated that abnormal neuronal inhibition during anesthetic exposure was responsible for the dramatic increase in apoptotic neurons,[47] more recent research[48]# seems to suggest that neuronal excitation, at least for some inhaled anesthetics, may be responsible for some of the deleterious effects in developing animals. The current findings of similar neurotoxic potency of equipotent clinically relevant doses of the three commonly used anesthetics seem to suggest that the phenomenon might be related to anesthetic potency, rather than inspired anesthetic concentration. Although the absolute concentrations of isoflurane and desflurane varied almost 5-fold, their neurotoxic potency, at least in the superficial neocortex, seemed comparable. Moreover, the qualitative distribution pattern of neuroapoptosis in other brain regions did not differ among the three different volatile anesthetics.

Yuede CM, Creeley CE, Olney JW: Caffeine augments neuroapoptosis induced in the infant mouse brain by isoflurane. Presented at the International Anesthesia Research Society Meeting and SAFEKIDS International Science Symposium, March 20, 2010, Honolulu, Hawaii.

Limitations

The small rodent model of prolonged anesthetic exposure used in the current study has several limitations. Because of the small respiratory tidal volume of these animals, the end-

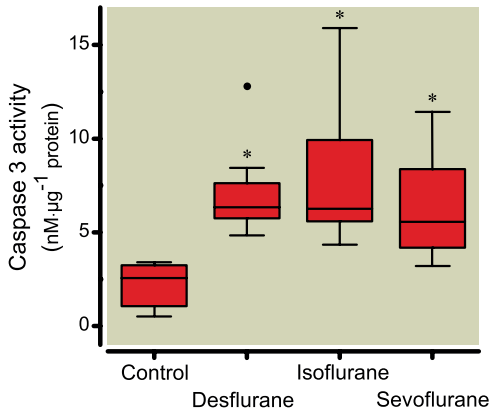

Fig. 5. Exposure to equipotent doses of desflurane, isoflurane, or sevoflurane similarly increases neocortical caspase 3 activity in neonatal mice. Box plots represent quantification of activity of the apoptotic enzyme caspase 3 in neocortical tissue using a colorimetric assay. Horizontal lines represent respective group medians; boxes, 25th–75th percentile; whiskers, 10th–90th percentile; and closed circle, outlier. The 7–8-day-old mouse pups were treated for 6 h with room air (control, n = 10), 7.4% desflurane (n = 13), 1.5% isoflurane (n = 11), or 2.9% sevoflurane (n = 11). *$P < 0.0005$ compared with control. There were no differences among the anesthesia groups ($P > 0.8$), by the Kruskal–Wallis test with the Dunn test for *post hoc* comparisons.

expiratory anesthetic concentration could not be measured and the investigation relied on anesthetic concentrations measured in the inspiratory gas mixture. Given the spontaneous respiration during the entire study period and the observed metabolic and respiratory derangements, anesthetic potency and, therefore, MAC could have varied during the 6-h exposure. In the current study, quantification of neuroapoptosis was limited to superficial layers of the neocortex; however, although other brain areas were not included in the cell counts, many seemed qualitatively similar after desflurane, isoflurane, or sevoflurane exposure, as demonstrated in figure 3.

The current study, similar to previous investigations,[39–41] used activated caspase 3 expression as the sole marker of neuronal degeneration. Although caspase 3 is the main cell death marker and executioner enzyme of the apoptotic cell death cascade,[37] it also has nonproteolytic function in some brain areas, such as the olfactory bulb, acting in the regulation of cell proliferation, differentiation, and inflammation.[49] Accordingly, we cannot exclude that some of the caspase 3–positive neurons may not complete the cell death cascade and survive because long-term neuronal density was not quantified in the current study. However, several lines of evidence lead us to believe that survival may only happen in a few caspase 3–positive cells, if at all. First, our group and others have previously verified that expression of other cell death markers that do not rely on caspase expression, such as Fluoro Jade B[7] or silver stain,[6,38] quantitatively and qualitatively reflect activated caspase 3 expression in the neocortex of neonatal mice and rats after anesthetic exposure. Second, in the current study, the cellular morphologic features of caspase 3–expressing cells during high-power magnification using confocal microscopy demonstrated a continuum of cell death ranging from degenerative changes, such as dendritic atrophy, dendritic beading, and cellular shrinkage, to cell death morphologic features, such as nuclear pyknosis, atrophy, and karyorrhexis (fig. 4), suggesting that most caspase 3–positive cells continued toward cellular death.

Similar to clinical practice, all three anesthetic agents were administered in 30% oxygen, to increase the safety margin to prevent hypoxia during anesthetic exposure. Because control animals were exposed to room air, the slightly higher oxygen concentration during the 6-h anesthetic might have led to oxygen toxicity, which could have contributed to increased caspase expression. However, previous studies[50] in neonatal rats have not observed any increase in neuronal cell death, even after 24-h exposure to 40% oxygen, suggesting that oxidative stress was not a significant contributor to the observed neuroapoptosis. In the current study, quantification of brain cell death was limited to neurons, as identified by NeuN staining. Unpublished data from our laboratory suggest that astrocytes and oligodendrocytes may not be susceptible to apoptotic cell death immediately after anesthetic exposure in 7-day-old mice. Although neocortical neuroapoptosis did not differ among the anesthesia groups, long-term neurologic outcome may be different and should, therefore, be part of future investigations. Researchers[7,46] have previously demonstrated that 6-h exposures to isoflurane or sevoflurane do not lead to a subsequent measurable detriment in learning and memory tasks in young adult mice using the Morris water maze.

In summary, 6-h exposures to equipotent clinically relevant doses of the three inhaled anesthetics (*i.e.*, desflurane, isoflurane, and sevoflurane) all dramatically increased neocortical neuronal apoptotic cell death in neonatal mice, without any quantitative or qualitative differences among the three anesthetics, suggesting that there may not exist any advantages to using any particular inhaled anesthetic over another regarding cytotoxic effects. However, these findings do not exclude that differential cytotoxicity might become apparent in other areas of the brain, during shorter exposure times, as recently observed in a comparison study of the anesthetic effects on dendritic architecture during exposure times lower than 1 h[51] or when using decreased doses.[46]

The clinical relevance of the current findings in newborn rodents remains uncertain. The clinical setting during pediatric surgery is dissimilar from anesthesia exposure in the animal laboratory and includes stressors, such as comorbidities, pain, surgical stimuli, and inflammatory responses, while omitting others, such as frequent mortality and derangement of physiologic factors observed in rodents.[52] Moreover, human epidemiologic studies suggesting neurologic abnormalities after surgery with anesthesia either did not specify the anesthetic drugs used[53,54] or used combinations of nitrous oxide, halothane, and ketamine.[55] Therefore, human outcomes after exposure to desflurane, isoflurane, or sevoflurane, the inhaled anesthetics commonly used in contemporary pediatric anesthesia practice and tested in the current study, remain unknown. Moreover, several human studies have not found any neurologic sequelae after anesthetic or sedative exposure early in life[56,57] or have suggested other factors unrelated to anesthetics, such as genetic vulnerability, as a causative factor for neurologic impairment after surgery.[58] However, the serious ramifications of potential neuronal cell death during, and long-term neurologic abnormalities after, anesthetic exposures early in life dictate continued research efforts, both clinically and preclinically, into this important controversy in pediatric anesthesiology.

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